

REPORT DOCUMENTATION PAGE			Form Approved OMB NO. 0704-0188		
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1. REPORT DATE (DD-MM-YYYY)		2. REPORT TYPE New Reprint		3. DATES COVERED (From - To) -	
4. TITLE AND SUBTITLE The Polysaccharide Capsule of Campylobacter jejuni Modulates the Host Immune Response			5a. CONTRACT NUMBER W911NF-12-1-0390		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER 611103		
6. AUTHORS A. C. Maue, K. L. Mohawk, D. K. Giles, F. Poly, C. P. Ewing, Y. Jiao, G. Lee, Z. Ma, M. A. Monteiro, C. L. Hill, J. S. Ferderber, C. K. Porter, M. S. Trent, P. Guerry			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAMES AND ADDRESSES University of Texas at Austin 101 East 27th Street Suite 5.300 Austin, TX 78712 -1539				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211				10. SPONSOR/MONITOR'S ACRONYM(S) ARO	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S) 61789-MA-MUR.4	
12. DISTRIBUTION AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.					
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15. SUBJECT TERMS Polysaccharide					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Claus Wilke
a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER 512-471-6028

Report Title

The Polysaccharide Capsule of *Campylobacter jejuni* Modulates the Host Immune Response

ABSTRACT

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REPORT DOCUMENTATION PAGE (SF298)
(Continuation Sheet)

Continuation for Block 13

ARO Report Number 61789.4-MA-MUR
The Polysaccharide Capsule of Campylobacter ...

Block 13: Supplementary Note

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2 **The polysaccharide capsule of *Campylobacter jejuni* 81-176 modulates the**
 3 **host immune response**

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5 Alexander C. Maue¹, Krystle L. Mohawk¹, David K. Giles^{*2}, Frédéric Poly¹, Cheryl
 6 P. Ewing¹, Yuening Jiao³, Ginyoung Lee³, Zuchao Ma³, Mario A. Monteiro³,
 7 Christina L. Hill¹, Jason S. Ferderber¹, Chad K. Porter¹, M. Stephen Trent⁴, and
 8 Patricia Guerry^{1#}

9 Enteric Diseases Dept., Naval Medical Research Center, Silver Spring, MD¹,
 10 Dept. of Biological and Environmental Sciences, University of Tennessee at
 11 Chattanooga, TN², Dept. of Chemistry, University of Guelph, Guelph, Ontario³
 12 and the Section of Molecular Genetics and Microbiology, University of Texas at
 13 Austin, Austin, TX⁴

14

15 #Corresponding author. Mailing address: Enteric Diseases Dept., Naval Medical
 16 Research Center, 503 Robert Grant Ave, Silver Spring, MD 20910; Telephone:
 17 301-319-7662; Email: patricia.guerry@med.navy.mil

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19 Running title: Immune modulation by *C. jejuni* capsule

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Abstract

Campylobacter jejuni is a major cause of bacterial diarrheal disease worldwide. The organism is characterized by a diversity of polysaccharide structures, including a polysaccharide capsule. Most *C. jejuni* capsules are known to be decorated non-stoichiometrically with methyl phosphoramidate (MeOPN). The capsule of *C. jejuni* 81-176 has been shown to be required for serum resistance, but here we show that an encapsulated mutant lacking the MeOPN modification, *mpnC*, was equally sensitive to serum killing as the non-encapsulated mutant. A non-encapsulated mutant, *kpsM*, exhibited significantly reduced colonization compared to wildtype 81-176 in a mouse intestinal colonization model, and the *mpnC* mutant showed an intermediate level of colonization. Both mutants were associated with higher levels of IL-17 expression from lamina propria CD4+ cells compared to cells from animals infected with 81-176. In addition, reduced levels of TLR-4 and -2 activation were observed following in vitro stimulation of human reporter cell lines with *kpsM* and *mpnC* compared to wildtype 81-176. The data suggest that the capsule polysaccharide of *C. jejuni*, and the MeOPN modification, modulate the host immune response.

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Introduction

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Campylobacter jejuni is one of the major causes of bacterial diarrhea worldwide. The organism is unusual among enteric pathogens in that it expresses a polysaccharide capsule (CPS) that contributes to serum resistance, invasion of intestinal epithelial cells in vitro and virulence in ferret and *Galleria mellonella* larvae models of disease (3, 8, 46). CPS is the major serodeterminant of the Penner serotyping scheme of *C. jejuni* (27) of which there are 47 serotypes, a reflection of the diversity of polysaccharide capsular structures in *C. jejuni*. In addition to variation in sugar composition, the CPS can be modified with ethanolamine, glycerol and O-methyl phosphoramidate (MeOPN). The MeOPN modification, which is found on about 75% of *C. jejuni* CPSs, has been shown to modulate cytokine release from mouse dendritic cells and to be a key determinant in virulence in the moth larvae model of disease (8). Both CPS expression itself (3) and expression of the modifications are phase variable due to slip strand mismatch repair (20, 28, 40). Thus, reversible phase variations in multiple genes result in mixed populations of wildtype cells, some of which express CPS and others that do not (3). Similarly, the levels of the MeOPN modifications found on the CPS are present in non-stoichiometric amounts because of phase variation in genes encoding the enzymes involved in transfer of these groups to specific sugars (37).

We have shown that a polysaccharide conjugate vaccine composed of the capsule of strain 81-176 conjugated to carrier protein CRM197 showed significant protection against diarrheal disease in a non-human primate model of

diarrhea, also suggesting a role for CPS in virulence (38). Here we further demonstrate that the polysaccharide CPS and the MeOPN modification both play significant roles in modulation of several aspects of the immune response, including serum resistance, activation of NF- κ B, and cytokine induction in vivo.

Materials and Methods

Bacterial strains and media. *C. jejuni* strain 81-176, its motile, isogenic *kpsM* and the complement of that mutant have been described (3). Bacteria were routinely cultivated microaerobically on Mueller Hinton (MH) agar supplemented with antibiotics as appropriate. For serum resistance assays, strains were grown in biphasic MH cultures for 18-20 h at 37°C. For mouse infection studies, strains were inoculated in MH broth to an OD₆₀₀ of ~0.01-0.05 and incubated with shaking in microaerobic conditions at 37°C for 18 hours.

Mutation and complementation of a gene for biosynthesis of MeOPN in *C. jejuni* 81-176. A region of the CPS locus of the 81-176 chromosome encoding genes for MeOPN synthesis (37) was cloned as a PCR fragment into BamHI-digested pBluescript. The primers used were pg08.90 (CGGGATCCGGAATGCCTGCTGTTATAGGAGTTGGA) in CJJ81176_1417 (labeled *mpnA* in Fig. 1) and pg08.91 (CGGGATCCCATCGAAGCATCATCTTCAACTTGAGC) in CJJ81176_1413 (*kpsC*). Both primers introduced a BamHI site at the 5' ends, as indicated by the underlining. The resulting plasmid was subjected to transposon mutagenesis using an in vitro Tn5-based transposition system (Epicentre, Madison, WI) with a

88 chloramphenicol resistance (Cm^r ; *cat*) cassette, and the insertion points were
 89 identified by sequencing individual insertions with primers within the cassette, as
 90 previously described (11, 19, 26). A clone with a non-polar insertion into gene
 91 CJJ81176_1415 (*mpnC* in Fig. 1) was identified; the insertion was 472 bp into
 92 the 762 bp gene. This plasmid was used to electroporate *C. jejuni* 81-176 to
 93 Cm^r , and the resulting mutant was confirmed to have undergone a double
 94 crossover by PCR with primers that bracketed the insertion point of the
 95 transposon. The mutant was complemented by cloning a wildtype allele of
 96 *mpnC* into pRY107/28, which is pRY107 (56) containing the σ^{28} promoter of *flaA*
 97 cloned between the XbaI and BamHI sites. The wildtype *mpnC* gene was PCR
 98 amplified using high fidelity polymerase (Clontech) and primers pg08.155 (5'-
 99 CCGGATCCGGTATAATGTGGCATATTGAAAGAG-3') and pg08.150 (5'-
 100 CCGCTCGAGCTCTTAATCATCTCCATCGAGATAAATAAG-3') that introduced
 101 BamHI and XhoI sites, respectively. This fragment was cloned into BamHI-XhoI
 102 digested pRY107/28. The plasmid complement was introduced into 81-176
 103 *mpnC* by conjugal transfer from *E. coli* DH5 α containing RK212.2, as previously
 104 described, with selection on kanamycin (18).

105 **^{31}P -Nuclear Magnetic Resonance (NMR) spectrometry.** Preparations
 106 containing the CPSs were dissolved in D_2O and ^{31}P NMR was performed on a
 107 400 MHz Bruker NMR instrument. Ortho-phosphoric acid was used as the
 108 external reference (δ 0.00).

109 **Serum survival assays.** 18 h cultures of *C. jejuni* grown in MH biphasic
 110 media were washed and adjusted to an OD_{600} 0.1 in MEM medium. Aliquots

111 (100 μ l) were added to wells of a 24-well plate containing 900 μ l of pre-warmed
 112 MEM media supplemented with 10% normal human serum (Sigma; NHS), and
 113 incubated under microaerobic conditions at 37°C. The percentage of survivors
 114 was determined by serial dilution onto MH agar plates. Assays were run in
 115 duplicate 3-4 times.

116 **Mouse infection experiments.** Seven- to eight-week old BALB/c mice
 117 (Jackson Laboratories, Bar Harbor, ME) were housed in groups of 10 with
 118 access to food and water ad libitum. For infection with *C. jejuni*, one liter of broth
 119 culture was harvested by centrifugation and resuspended in PBS. The inocula
 120 were normalized by OD₆₀₀ to $\sim 10^{11}$ CFU/mL, and animals were inoculated
 121 intragastrically with 100 μ L of the cell suspension. The inocula doses were
 122 validated on MH agar plates prior to and immediately after infection of the
 123 animals.

124 **Assessment of colonization.** Fecal collections were performed by
 125 allowing individual mice to defecate in clean, empty shoebox cages prior to
 126 returning to group housing. Feces were collected using forceps into 5 mL Falcon
 127 snap-cap tubes and then diluted 1:10 by weight into PBS. Various stool dilutions
 128 were plated onto campylobacter selective media (CVA plates; Remel) and
 129 incubated under microaerobic conditions at 42°C for 2 days.

130 **Lymphocyte isolation.** At indicated time points following oral challenge,
 131 mice were sacrificed and small intestines were removed to recover lymphocytes
 132 from the lamina propria (LPL; lamina propria lymphocytes) as described

133 previously (32) with some modifications. In brief, Peyer's patches (PP) were
 134 removed from the intestines and intestines were cleared of contents using
 135 forceps, opened longitudinally and then cut into ~5mm sections. Intraepithelial
 136 lymphocytes (IEL) were removed from these intestinal sections by placing the
 137 tissue in a solution of 1 mM DTT/1 mM EDTA at 37°C for two 20 minute
 138 incubations. After each incubation, the supernatant was removed and replaced
 139 with fresh DTT/EDTA. To isolate LPLs, the remaining intestinal pieces were
 140 digested with collagenase D (Roche, 1 mg/ml) and DNase I (Sigma, 40 µg/ml) for
 141 two 1 h incubations at 37° C. The supernatant was removed following each
 142 incubation and replaced with fresh media. Following the digestion of small
 143 intestinal tissue sections, cells were pelleted by centrifugation and LPLs were
 144 isolated using a discontinuous (80-40%) Percoll gradient.

145 **Intracellular cytokine staining.** LPL were cultured *in vitro* for 4-6 hours in
 146 the presence of media alone or phorbol-12-myristate-13-acetate (PMA) (Sigma,
 147 20 ng/ml) and ionomycin (Sigma, 0.5µg/ml). Media was DMEM supplemented
 148 with 10% FBS, 2mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, non-
 149 essential amino acids, 50 µM 2-mercaptoethanol, penicillin (100 IU/ml) and
 150 streptomycin (100 µg/ml). Protein transport was inhibited with the addition of
 151 brefeldin A (10µg/ml). Following culture, LPLs were stained with CD4 APC.
 152 Cells were fixed in 4% formaldehyde prior to permeabilization with 0.1% saponin
 153 (in PBS and 1% FBS). Intracellular staining was performed using anti-mouse
 154 IFN-γ FITC (eBioscience) or anti-mouse IL-17 PE (eBioscience). Cells were then
 155 washed and resuspended in 1% formaldehyde prior to analysis on a Becton

156 Dickinson FACScan equipped with red and blue lasers (i.e. 5-color capability).

157 Data were analyzed using FlowJo software (TreeStar).

158 **TLR signaling assay using whole bacteria.** The following cell lines
 159 were purchased from InvivoGen: HEK-Blue-hTLR4, HEK-Blue-hTLR2 and THP1-
 160 XBlueTM-MD2-CD14. The human epithelial kidney (HEK) 293 cells are stably
 161 transfected with either human TLR4, MD2 and CD14 (HEK-Blue-hTLR4) or
 162 human TLR2 and CD14 (HEK-Blue-hTLR2). THP1-XBlueTM-MD2-CD14 cells
 163 are derived from the human monocytic THP-1 cell line and are stably transfected
 164 with MD2 and CD14. These HEK-Blue and THP1-XBlue clones also stably
 165 express secreted embryonic alkaline phosphatase (SEAP) under the control of a
 166 promoter inducible by NF- κ B and activator protein 1 (AP-1). Thus, stimulation of
 167 toll-like receptors will result in an amount of extracellular SEAP in the
 168 supernatant that is proportional to the level of NF- κ B induction. The HEK cell
 169 lines were maintained in standard DMEM with 10% heat-inactivated fetal bovine
 170 serum (FBS) (Gibco) supplemented with 4.5 g/L glucose, 2mM L-glutamine, 50
 171 U/mL penicillin, 50ug/ml streptomycin, 100ug/ml Normocin (InvivoGen) and 1X
 172 HEK-Blue selection (InvivoGen) in a 5% saturated CO₂ atmosphere at 37°C.
 173 The THP1 cell line was maintained in standard RPMI 1640 medium with 10%
 174 heat-inactivated fetal bovine serum (FBS) (Gibco) supplemented with 4.5 g/L
 175 glucose, 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 10mM HEPES, 1mM
 176 sodium pyruvate, 50 U/mL penicillin, 50ug/ml streptomycin, 100ug/ml Normocin
 177 (InvivoGen), 200ug/ml Zeocin (InvivoGen) and 250 ug/ml G418 (InvivoGen) in a
 178 5% saturated CO₂ atmosphere at 37°C.

179 The induction of TLR signaling in HEK-Blue-hTLR4, HEK-Blue-hTLR2 and
 180 THP1-XBlueTM-MD2-CD14 clones was assessed by measuring SEAP activity
 181 using QUANTI-BlueTM colorimetric assay (InvivoGen). The assays were
 182 performed according to manufacturer's protocols. Briefly, cells were seeded in a
 183 96-well plate in triplicate (2.5 X 10⁴ cells/well for HEK-Blue-hTLR4, 5 X 10⁴
 184 cells/well for HEK-Blue-hTLR2 and 1 X 10⁵ cells/well for THP1-XBlueTM-MD2-
 185 CD14). Whole bacterial cells were grown on Mueller Hinton agar for 16 h,
 186 collected from the plate, washed and resuspended in sterile PBS. 10-fold serial
 187 dilutions were prepared based on OD₆₀₀ to yield the number of bacteria
 188 inoculated into each well. CFU were confirmed by plating serial dilutions on MH
 189 agar. Erythromycin (50 ug/ml) was included to prevent bacterial growth during
 190 incubation, and other antibiotics used in the media for cell propagation were
 191 omitted in the assay. After 18 h incubation, supernatants (20ul) were transferred
 192 to a 96- well plate and incubated at 37°C with QUANTI-Blue (180ul). SEAP
 193 activity was measured by reading OD₆₅₅ nm with a Synergy Mx multi-mode
 194 microplate reader (BioTek).

195 **Statistical analyses.** Differences in mouse colonization level, as
 196 assessed by the number of organisms shed (log 10 of CFU/gram feces), were
 197 compared using a repeated measures analysis of variance with the *C. jejuni*
 198 strain as the between animal factor (i.e., wild type, *kpsM* mutant, *mpnC* mutant)
 199 and collection time-points as the repeated factor. The covariance structure was
 200 modeled using a first-order antedependence model. A Tukey adjustment was
 201 utilized to control the type I error rate. Comparisons of the proportion of mice

202 infected by strains over time were made using a cox proportional hazards model.
 203 These analyses were conducted with SAS version 9.2 for Windows (SAS
 204 Institute, Inc., Cary, North Carolina) using a two-tailed alpha of 0.05.

205 Statistical analyses of complement killing, intracellular cytokine
 206 expression, and TLR assays were analyzed using student's t test. Differences
 207 were considered significant at $P < 0.05$.

208 **Results**

209 **Construction of a mutant in the MeOPN biosynthetic pathway of 81-**

210 **176.** McNally et al. (37) identified the genes in *C. jejuni* strain NCTC 11168
 211 (CJ1415-18) that were responsible for MeOPN synthesis, as well as two distinct
 212 MeOPN transferases that were responsible for attachment of MeOPN to two
 213 different sites in the polysaccharide CPS of this strain. CJ1415-CJ1418 are
 214 highly conserved among *C. jejuni* strains, while the transferases are more
 215 variable based on differences in attachment of the MeOPN to sugars. The genes
 216 corresponding to CJ1415-1418 in 81-176 are CJJ81176_1414-CJJ81176_1417.
 217 Since the function of genes has been established in NCTC 11168, we have
 218 named the genes for MeOPN synthesis *mpnA-D*, shown in Fig. 1A, for clarity in
 219 discussing these conserved genes in different strains. A mutant in *mpnC* in 81-
 220 176 was shown to lack MeOPN by ^{31}P -NMR, as predicted based on the NCTC
 221 11168 data (37), and MeOPN was restored when the mutant was complemented
 222 (Fig. 1). The *mpnC* mutant produced CPS as determined by both NMR and

223 immunoblotting with rabbit polyclonal antibody to whole cells of 81-176 (data not
224 shown).

225 **MeOPN contributes to serum resistance.** There have been several
226 reports demonstrating that non-encapsulated mutants of *C. jejuni* are more
227 sensitive to normal human serum than wildtype strains (3, 29). Comparable data
228 to those published for 81-176 and its isogenic *kpsM* mutant (3) are shown in Fig.
229 2. Surprisingly, the *mpnC* mutant, expressing the polysaccharide CPS lacking
230 MeOPN, displayed the same pattern of serum killing as the *kpsM* mutant lacking
231 all CPS (Fig. 2). The *mpnC* mutant was significantly more sensitive than wildtype
232 to complement at both 60 min ($P < 0.001$) and 120 min ($P < 0.005$). When the
233 *mpnC* mutant was complemented in trans, serum resistance returned to levels
234 comparable to wildtype (Fig. 2).

235 **Capsule is required for prolonged mouse colonization.** The ability of
236 the *kpsM* and *mpnC* mutants to colonize mice was compared to wildtype in a
237 series of experiments. Animals were intragastrically infected with *C. jejuni* and
238 colonization was monitored post-infection by fecal shedding. Following infection,
239 wildtype *C. jejuni* 81-176 colonized mice on average at levels exceeding 10^6
240 CFU/g feces (Fig. 3A). This high level of colonization was maintained for greater
241 than 15 days before counts began to drop below the initial colonization levels.
242 Mice infected with the *kpsM* mutant generally had early colonization levels similar
243 to that of wildtype (Fig. 3A). In addition, the *kpsM*-infected mice had a shorter
244 duration of colonization compared to those infected with wildtype ($P = 0.06$). The
245 majority of *kpsM*-infected mice cleared the infection by day 18 in comparison to

the wildtype-infected mice that remained colonized at some level through day 28 post-infection (the last day tested) (Fig. 3A). Thus, despite the similar level of colonization seen early post-infection, by day 14 there was a statistically significant difference in colonization levels of mice infected with *kpsM* when compared to wildtype ($P < 0.01$).

In parallel experiments, colonization capacity of the *mpnC* mutant was compared to wildtype. Upon infection with the *mpnC* mutant, mice shed similar numbers of *C. jejuni* in their stool in comparison to wildtype (Fig. 3B). In fact, there were no significant differences in stool counts between the *mpnC* mutant and wildtype during the first 10 days post-infection, and only later in infection did the *mpnC* mutant demonstrate a significant reduction in the level of colonization when compared to wildtype (Fig. 3B; $P = 0.02$ for day 20). Thus, although not directly compared, the colonization ability of the *mpnC* mutant appeared intermediate in nature compared to wildtype and *kpsM* (Fig. 3).

IL-17 expression from intestinal T cells is modulated by the polysaccharide CPS. To determine if CPS had a role on immune responses in vivo, mice were orally infected with wildtype 81-176 and *mpnC* or *kpsM* strains. At selected times post-infection, T cells were isolated from small intestine Peyer's patches, epithelium and lamina propria. Following an ex vivo restimulation, the expression of IL-17 and IFN- γ were determined using intracellular cytokine staining and flow cytometry. Figure 4A shows representative histograms for CD4 LPLs and dot plots demonstrating gating strategies for intracellular cytokine staining (Fig. 4B). Following infection with either *kpsM* or wildtype strains, CD4

269 cells from the lamina propria of *kpsM*-infected mice exhibited significantly higher
270 percentages ($P < 0.05$) of CD4⁺ cells that expressed IL-17 at day 7 and day 21
271 (Fig 4C). No difference was observed in IFN- γ expression of CD4⁺ LPLs isolated
272 from *kpsM* or wildtype infected mice at either timepoint. Differences in cytokine
273 expression were not observed from PP cells or IELs (data not shown).

274 Next, the MeOPN modification on CPS was examined for its role in
275 modulating immune responses in vivo. CD4⁺ LPL from *mpnC*-infected mice did
276 not express significantly higher percentages of IL-17⁺ cells on day 7 compared to
277 LPL isolated from mice orally challenged with wildtype 81-176 (Fig 4D).
278 However, on day 21 post-infection, mice colonized by *mpnC* exhibited
279 significantly higher percentages ($P < 0.01$) of CD4⁺ LPLs expressing IL-17 than
280 animals infected with wildtype. In addition, no significant differences were seen
281 in other lymphocyte subsets or in IFN- γ expression patterns (data not shown).

282 **Effects of CPS on TLR-signalling.** To determine the impact of CPS
283 production and modification on TLR activation, we performed reporter cell
284 signaling assays with whole bacteria. The *kpsM* mutant exhibited significantly
285 higher activation than wildtype from 10^4 to 10^7 CFU for hTLR4 activation and from
286 10^5 - 10^8 CFU for hTLR2 activation (Fig. 5). Although similar results were seen
287 with the *mpnC* mutant, the lack of complete complementation confounds these
288 results (data not shown).

289 We subsequently compared the overall TLR-receptor activation of *kpsM*
290 and *mpnC* mutants using a human monocytic reporter line that expresses several
291 TLRs, including TLR1, TLR2, TLR4, TLR6, TLR8, NOD1 and NOD2. Significant

292 increases ($P < 0.005$) in signaling were observed for both mutant strains
 293 compared to wildtype and their complements (Fig. 6). For the *kpsM* mutant
 294 these differences were observed from 10^4 - 10^7 CFU and for the *mpnC* mutant the
 295 differences were observed between 10^5 - 10^7 CFU.

296

297 Discussion

298 *C. jejuni* remains a poorly understood pathogen, in part because of the
 299 absence of small animal models that mimic human disease. Following orogastric
 300 infection with *C. jejuni*, adult, immunocompetent mice can become colonized for
 301 variable lengths of time, but without the disease symptoms seen in
 302 immunodeficient mice (5, 6, 12, 23, 35, 39). Despite the lack of disease, the
 303 mouse model can provide information on traits required for colonization, the first
 304 step in pathogenesis (39). Here we show that wildtype 81-176 colonizes BALB/c
 305 mice better than either an isogenic mutant lacking capsule (*kpsM*) or a mutant
 306 expressing CPS without MeOPN (*mpnC*). Interestingly, a reduction in
 307 colonization ability of a MeOPN-mutant of 81-176 (in the gene corresponding to
 308 *mpnA*) compared to wildtype 81-176 was also reported in MyD88-defective mice
 309 (54). In our studies BALB/c mice that were colonized with wildtype 81-176
 310 remained colonized for the duration of the experiments (>21 days). The *kpsM*
 311 mutant showed similar colonization levels for about 9 days before colonization
 312 levels dropped. The *mpnC* mutant colonized at levels that were generally lower
 313 than wildtype, although reaching statistical significance only at day 20. Thus,

314 expression of CPS by *C. jejuni* facilitated colonization in the mouse model. CPS
315 has also been shown to play a role in *C. jejuni* colonization of chickens (2, 16).

316 Following restimulation, IL-17 production by CD4⁺ LPLs was reduced in
317 mice colonized by *C. jejuni* 81-176 compared to both mutant strains. Mice
318 colonized by either the *kpsM* or *mpnC* mutants possessed higher levels of IL-17⁺
319 CD4⁺ cells in the small intestine compared to wildtype at day 21, and this
320 increased IL-17 production was associated with a reduction in colonization levels.
321 However, despite the fact that both the *kpsM* mutant and the *mpnC* mutant were
322 associated with higher frequencies of IL-17-producing CD4⁺ cells in the small
323 intestine compared to wildtype 81-176, the *kpsM* mutant appeared to show a
324 greater reduction in colonization capacity than the *mpnC* mutant suggesting that
325 the presence of the polysaccharide CPS, even without the MeOPN modification,
326 affords some protection against the immune response in the intestine. Although,
327 in vivo cytokine responses were not measured directly in this study, the data
328 suggest that CPS expression, and more specifically, the MeOPN modification on
329 the wildtype capsule, may affect the generation of IL-17 responses in the gut
330 mucosa. Future studies are needed to determine the specificity of the IL-17
331 response against *C. jejuni* in the intestine.

332 T helper-17 (Th17) responses have come into focus due to their roles in
333 maintaining intestinal homeostasis (24, 50) and protective immune responses
334 against enteric pathogens (15, 36, 49, 50). The gut Th17 response is composed
335 of both innate and adaptive immune system components. Innate Th17 (iT17)
336 responses are induced by segmented filamentous bacteria (SFB) that colonize

the gut (13, 25) and maintain a symbiotic balance between the microbiota and host (50). Specific animal vendors supply mice that are either colonized with SFB or not (Jackson Laboratories, SFB⁻; Taconic SFB⁺) (25). These models can be exploited to evaluate innate or adaptive Th17 responses. Whereas iTh17 responses can be induced relatively quickly by cytokine signals (15, 36), adaptive Th17 responses occur later (days to weeks) (47) and are antigen-specific responses. In our present study, the Th17 responses likely represent an adaptive immune response since Jackson Laboratory mice were used. Key to Th17 responses are the cytokines interleukin-17 (IL-17) and IL-22 (29, 36, 39, 47), and the upstream cytokines that lead to their expression such as IL-1, IL-6, and IL-23 (31, 33, 47). IL-17 is primarily thought to be effective against extracellular pathogens by inducing inflammation and recruiting neutrophils to sites of infection (reviewed in (31)). IL-22 exerts its protective effects by inducing epithelial cells to produce anti-bacterial molecules (57). Recently, Th17 responses have been demonstrated to have protective roles against *Salmonella* and *Citrobacter* infections in mice (15, 36). To date only limited data exist regarding Th17 responses and *Campylobacter* infection. Edwards et al. (10) showed that cytokines involved in Th17 responses were induced in colon biopsy tissues following co-culture with *C. jejuni* and that the addition of exogenous IL-17 reduced *C. jejuni* invasion into an intestinal epithelial cell line. However, additional work must be performed to determine the precise role of Th17 immune responses to *C. jejuni*.

359 Consistent with these data, we have also shown that the presence of the
 360 CPS on wildtype 81-176 resulted in reduced activation of both TLR2 and TLR4
 361 using HEK cells expressing each receptor. Our data are consistent with those of
 362 Rose et al. (46) who showed that mutants of NCTC 11168 lacking CPS or
 363 MeOPN induced higher levels of IL-6, TNF α and IL-10 from mouse dendritic cells
 364 compared to the wildtype strain. Using dendritic cells from TLR4^{-/-} mice, they
 365 also showed that some of these differences in cytokines were due to TLR4
 366 signalling. Similar down-regulation of the immune response has been observed
 367 for other bacterial capsules (9, 42-44, 48, 51). In some cases this inhibition may
 368 be due to shielding of the bacterial surface by the capsule and prevention of TLR
 369 stimulation. However, the CPS of *Neisseria meningitidis* actively inhibits TLR2
 370 activation by binding CD14 (30). The MeOPN modification on two distinct CPS
 371 structures of *C. jejuni* has now been shown to modulate cytokine responses and
 372 TLR signaling (46), suggesting an active role for this unusual structure. Similarly,
 373 the *C. jejuni* CPS may inhibit binding of complement activators and components
 374 to the surface of the bacterial cell, but the fact that the *mpnC* mutant was as
 375 sensitive as the *kpsM* mutant to complement killing, also suggests an active role
 376 for the MeOPN group. In contrast to *C. jejuni*, modification of *Hemophilus*
 377 *influenzae* lipopolysaccharide with phosphorycholine, which is also under phase
 378 variable expression, enhances sensitivity to complement killing (55). The
 379 mechanism by which MeOPN interacts with components of the complement
 380 cascade is under investigation.

381 Collectively, these data indicate the polysaccharide CPS of 81-176, and
 382 the MeOPN modification, modulate the immune response to this pathogen and
 383 are consistent with previous observations suggesting a stealth strategy by which
 384 *C. jejuni* may avoid the immune response. It has been known for some time that
 385 *C. jejuni* flagellin is unable to induce TLR5 because of structural changes to the
 386 monomeric subunit protein that are reflected in changes in filament formation (1,
 387 14). *C. jejuni* also expresses altered linkages of hydroxyacyl chains on lipid A
 388 that reduce TLR4 activation (52), and there is evidence that the *N*-linked glycan
 389 on proteins and certain LOS glycoforms can down regulate IL-6 induction (53).
 390 Previous work has shown that the CPS of NCTC 11168, and specifically the
 391 MeOPN modification on this CPS, reduced cytokine production from mouse
 392 dendritic cells in culture (46). Here, we have demonstrated that a second *C.*
 393 *jejuni* CPS and the MeOPN modification modulates the immune response at
 394 multiple levels, including resistance to complement killing and cytokine induction
 395 via NF- κ B signalling. The ability to avoid the immune response of the host
 396 provides an advantage in establishing colonization by *C. jejuni*, be it as a
 397 commensal in animals or as a pathogen in humans. Moreover, asymptomatic
 398 infection by *C. jejuni* is common among children in the developing world and
 399 acute infections are frequently followed by periods of asymptomatic shedding (7,
 400 41, 45), which may be due, at least in part, to the ability of this pathogen to avoid
 401 the host immune response. Similarly, recrudescence of infection following
 402 appropriate antibiotic treatment in an immunocompetent adult has been reported
 403 (4).

404 One of the hallmark characteristics of *C. jejuni* is its ability to undergo
 405 phase variation of surface antigens by slip strand mismatch repair (3, 17, 21, 22,
 406 34, 40). In terms of the polysaccharide CPS, this phase variation occurs at two
 407 levels. One is the high frequency on/off reversible expression that was originally
 408 described in strain 81-176, such that a culture grown in vitro is a mixed
 409 population of encapsulated and unencapsulated variants (3). The other level of
 410 phase variation affects CPS structure and is best understood in terms of the
 411 MeOPN modification. Thus, all MeOPN transferases that have been described
 412 to date are subject to phase variation at homopolymeric tracts of bases, resulting
 413 in non-stoichiometric amounts of this modification. The reason for this variability
 414 in both CPS expression and structure is not understood, but the data presented
 415 here suggest that the polysaccharide CPS, with and without MeOPN, modulates
 416 the host immune response at multiple levels. Since *C. jejuni* produces an
 417 inflammatory diarrhea, phase variation during replication in vivo may also
 418 modulate the severity of illness, and, at least in part, explain variability in severity
 419 of symptoms seen with this pathogen.

420 **Acknowledgements**

421 Work at NMRC was funded by NIAID R56 AI080593 (to PG) and Navy
 422 Work Unit 6000.RAD1.DA3.A0308. Work at UT Austin was funded by NIAID
 423 grants AI064184, AI76322 and ARO Grant 61789-MA-MUR (to MST). Work at
 424 U of Guelph was funded by NSERC.

425 We thank Dawn Pattarini for technical assistance. The views expressed in
 426 this article are those of the authors and do not necessarily reflect the official
 427 policy or position of the Department of the Navy, Department of Defense, nor the
 428 US government. All animal experiments were conducted in compliance with the
 429 Animal Welfare Act and in accordance with the principles set forth in the "Guide
 430 for the Care and Use of Laboratory Animals," Institute of Laboratory Animals
 431 Resources, National Research Council, National Academy Press, 1966. PG and
 432 CKP are employees of the US government. This work was prepared as part of
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 436 member or employee of the US government as part of that person's official
 437 duties.

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Figure legends

Fig. 1. A. Cartoon of the capsule locus of 81-176. The locus is organized, like other class 2 capsule loci, into conserved regions 1 and 3 encoding proteins involved in capsule assembly and transport and the variable region 2 encoding proteins involved in polysaccharide synthesis. The MeOPN biosynthesis genes (*mpnA-D*) are found within region 2, but are highly conserved among strains if present and correspond to CJ1415-1418 in NCTC 11168 (37). Genes corresponding to CJJ81176_1418 and CJJ81176_1419 are also highly conserved among strains expressing MeOPN, but mutational analyses have failed to demonstrate a role for these genes in MeOPN synthesis (32). CJJ81176_1420 is annotated as a putative MeOPN transferase. There are 15 additional genes within region 2 of 81-176. B. ³¹P-NMR of CPS from wildtype 81-176 (blue), the *mpnC* mutant (green), and the *mpnC* mutant complemented in trans (red).

Fig. 2. Sensitivity of 81-176 and mutants to complement killing by NHS. The % survivors are shown after 60 and 120 minutes incubation with 10% NHS. The colors represent: black, 81-176; red, *kpsM*; green, *mpnC*; blue, *mpnC* complemented in trans.

Fig. 3. Colonization of BALB/c mice by *C. jejuni* strain 81-176 and various CPS mutants. The log CFU/g feces shed by wildtype and either a *kpsM* mutant (A) or an *mpnC* mutant (B) are shown over the course of infection. Groups of 10 mice were intragastrically infected with ~10¹⁰ CFU. Each data point represents

an individual mouse infected with wildtype (squares) or mutant (circles), and the group mean is displayed as a connected line (black for wildtype and dotted for mutants). The limit of detection was 10^2 CFU/g feces. These data are representative of 3-4 independent experiments.

Fig. 4. IL-17 expression is reduced in small intestinal CD4+ LPLs from mice infected with wildtype *C. jejuni* 81-176. BALB/c mice (4-5/group) were orally infected with $\sim 10^{10}$ CFU *C. jejuni*. At days 7 and 21 post-infection, small intestines were removed and processed to isolate LPLs. LPLs were restimulated in vitro with PMA (20 ng/ml) and ionomycin (500 ng/ml) for 4-6 h. Protein transport was inhibited by addition of Brefeldin A (10 μ g/ml). Intracellular cytokine staining for IL-17 and IFN- γ was performed on cells and analyzed by flow cytometry. (A) Representative histograms demonstrating the percentage of CD4+ and CD8+ cells isolated from mouse small intestines. (B) Representative dot plots demonstrating intracellular staining for IL-17 and IFN- γ in unstimulated and stimulated CD4+ LPLs. (C) Percent expression of IL-17 in CD4+ LPLs from mice infected with *kpsM* or wildtype *C. jejuni*. (D) IL-17 expression in CD4+ LPLs from mice infected with *mpnC* or wildtype *C. jejuni*. Data represent the mean \pm SEM. * indicates $P < 0.05$ and ** indicates $P < 0.01$ using student's t test. Data are representative of 2-3 independent experiments.

Fig. 5. Activation of (A) TLR4 in HEK-293 cells transfected with human TLR4-MD2-CD14 and (B) TLR2 in HEK-293 cells stably transfected with human TLR2-CD14. TLR activation was monitored colorimetrically using a

716 SEAP reporter gene placed under the control of an NR- κ B inducible promoter.
 717 Ten-fold serial dilutions of whole bacterial cells of the indicated strains of *C. jejuni*
 718 were added to each well in triplicate. Values represent the means and standard
 719 deviations of one experiment assayed in triplicate. The figure is representative of
 720 three independent experiments. Asterisks indicate P value <0.005 compared to
 721 wildtype 81-176 or the complement. Black lines, wildtype 81-176; red lines, the
 722 isogenic *kpsM* mutant; and blue lines, the *kpsM* mutant complemented in trans.

723 **Fig. 6. Activation of toll-like receptors using a human monocytic (THP-1)**
 724 **reporter cell line by *kpsM* (A) and *mpnC* (B) mutants compared to wildtype**
 725 **81-176 and complements of each mutant.** Bacteria were added to cells as
 726 described in the legend to Fig. 5. Values represent the means and standard
 727 deviations of one experiment assayed in triplicate. The figure is representative of
 728 three independent experiments. Asterisks indicate P value <0.005 compared to
 729 wildtype 81-176 or the complement. Black lines, wildtype 81-176; red line, the
 730 isogenic *kpsM* mutant; green line, the isogenic *mpnC* mutant; blue lines, the
 731 complement of each mutant.

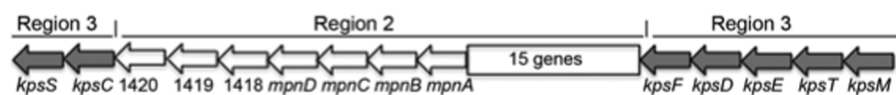
732

733

734

735

A



B

mpnC

Complement

81-176

